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TECHNICAL NOTE

An in vitro approach to the study of macula densa-mediated glomerular hemodynamics

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Tubuloglomerular feedback (TGF), which operates between the tubule and the parent glomerulus, is important to renal autoregulation and homeostasis of body fluid and electrolytes [1, 2]. Since the juxtaglomerular apparatus (JGA) displays an intimate anatomical relationship between the specialized tubular epithelial cells (macula densa) and the vasculature (afferent and efferent arterioles), it has long been suggested as the anatomical site of TGF [3]. However, attempts to obtain direct evidence to support this have been hindered by the anatomical complexity of the JGA. Since the JGA is located beneath the tubular layer at some distance from the surface of the kidney, the macula densa is not accessible to direct micropuncture in vivo, nor is direct observation of the vascular pole possible.

To circumvent these limitations of the in vivo approach, we have developed an in vitro preparation in which both the afferent arteriole and the macula densa of a microdissected rabbit JGA are microperfused simultaneously. This allows us to study the JGA directly without being influenced by systemic hemodynamic and hormonal factors; moreover, real-time images of the afferent arteriole can be continuously monitored and recorded. Unlike other preparations, there are only a few structures to interfere with direct observation of the vascular pole, permitting accurate measurement of the luminal diameter of the arterioles even in segments which are overlapped by the glomerulus. Using this preparation, we have observed that increasing the NaCl concentration of the macula densa perfusate constricts the afferent arteriole in the segment close to the glomerulus, and that this constriction is abolished by furosemide, a loop diuretic known to inhibit TGF.

Methods

Isolation and microperfusion of an afferent arteriole with macula densa

We used a method similar to that described in our previous papers to isolate the JGA [4, 5]. Briefly, young male New Zealand white rabbits (1.0 to 1.2 kg) fed standard rabbit chow (Ralston Purina Co., St. Louis, Missouri, USA) and tap water ad libitum were anesthetized with intravenous sodium pentobarbital (40 mg/kg) and given an intravenous injection of heparin (500 U). The aorta was catheterized below the renal arteries and clamped with a hemostat above the kidneys. The kidneys were perfused with cold medium 199 (Gibco Laboratories, Grand Island, New York, USA) containing 5% bovine serum albumin (BSA), then removed and sliced along the corticomedullary axis. Slices were placed in ice-cold medium 199 containing 5% BSA (medium 199-5% BSA) and microdissected under a stereomicroscope (SZH; Olympus, Overland Park, Kansas, USA) at magnifications up to 100×, using thin steel needles and sharpened forceps (No. 5, Dumont; Fine Science Tools, Inc., Belmont, California, USA).

An interlobular artery was removed from the remainder of the slice by grasping it at its origin from the arcuate artery and gently pulling it out together with the adhering tubular element. By illuminating this tissue from various directions, we were able to observe the afferent arteriole, its glomerulus and associated tubular segment that consists of the thick ascending limb, macula densa and distal convoluted tubule. Other tubular fragments were gently stripped off, taking care to avoid distortion of the afferent arteriole, glomerulus and the associated tubular segment or touching them with the forceps or needle. Once an appropriate preparation was obtained, the afferent arteriole was severed from the interlobular artery by cutting it with a mini-blade (George Tieman & Co., Plainview, New York, USA). We found that it is important to make the cut only once and precisely at a right angle to the afferent arteriole (90°) if subsequent cannulation is to be successful. Using a micropipette, the final preparation was transferred to a temperature-regulated chamber which was previously mounted on the stage of an inverted microscope (IMT-2; Olympus).

The method of cannulating the afferent arteriole was similar to that reported by Osgood et al [6] and employs the array of pipettes shown in Figure 1. The afferent arteriole was drawn into the holding pipette that had a constriction (internal diameter, 14 μm). The tip of the perfusion pipette (internal diameter, 8 μm) was advanced into the lumen and perfusion was begun. It should be pointed out that the tip of the perfusion pipette must be centered precisely to assure successful advancement into the arteriole. After perfusion was begun, we confirmed that the perfusate did not leak into the bath from the proximal end of the arteriole by the following method. While the pressure pipette (outer diameter at the tip, 2 μm), which was filled with NaCl solution containing 5% FD&C green and 4% KCl (pH 7; 300 mOsm/kg), was still within the perfusion pipette, the dye in the pressure pipette was injected and its movement observed. After

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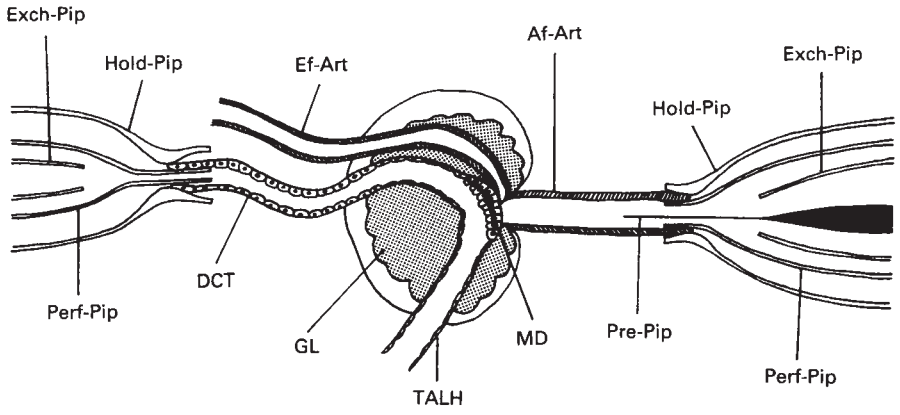

Fig. 1. *Schematic representation of the pipette arrangement used for perfusion of an afferent arteriole (Af-Art) and attached macula densa (MD).* Abbreviations are: TALH, thick ascending limb of Henle's loop; GL, glomerulus; DCT, distal convoluted tubule; Ef-Art, efferent arteriole; Hold-Pip, holding pipette; Perf-Pip, perfusion pipette; Exch-Pip, exchange pipette; Pre-Pip, pressure pipette.

ruling out leakage, the pressure pipette was advanced into the afferent arteriole through the opening of the perfusion pipette. The pressure pipette was observed under the microscope during application of various internal pressures. The pressure at which the colored solution did not flow into the afferent arteriole nor the intraluminal fluid into the pressure pipette was taken as being equal to the pressure in the afferent arteriole. This method, known as Landis technique, was used since it has been reported that pressure measurement by a servo-null system is not accurate with this arrangement of pipettes [6]. To facilitate exchange of the arteriolar perfusate, an exchange pipette was laid between the pressure and perfusion pipettes so that its tip was positioned near that of the perfusion pipette. Through this exchange pipette, the perfusion solution can readily be introduced into the perfusion pipette, and, if desired, the composition of the arteriolar perfusate can easily be changed. The perfusate was oxygenated medium 199-5% BSA, and the driving force was provided by a pressurized tank of 95% O_2 and 5% CO_2 attached to the proximal end of the perfusion pipette. An air regulator positioned between the tank and the perfusion pipette was manipulated to control intraluminal pressure as measured with the pressure pipette.

We next cannulated the tubular segment using a method similar to that originally described by Burg [7]. Briefly, the end of either the thick ascending limb of the loop of Henle or the distal convoluted tubule was drawn into a holding pipette which had a constriction (internal diameter, 14 μm), and the perfusion pipette (internal diameter, 7 μm) carefully advanced. Microperfusion was started with a modified Krebs-Ringer bicarbonate buffer of the following composition: 115 mM NaCl, 15 mM $NaHCO_3$, 10 mM sodium acetate, 0.96 mM NaH_2PO_4, 0.24 mM Na_2HPO_4, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose (total osmolality, 280 mOsm/kg). The tubular effluent flowed into the bath without being collected. The driving force used to maintain the tubular perfusion rate (about 10 nl/min) was provided by hydrostatic pressure.

The bath was identical to the arteriolar perfusate and was exchanged continuously. Microdissection and cannulation of both the afferent arteriole and tubular segment were completed within 90 minutes at 8°C, after which the temperature of the bath was gradually raised to 37°C for the rest of the experiment. Once it had stabilized, a 30-minute equilibration period was allowed before taking any measurements. When the temperature reached about 36°C, the arteriole exhibited spontaneous cyclic contractions which disappeared during equilibration, allowing us to measure the stable arteriolar lumen diameter.

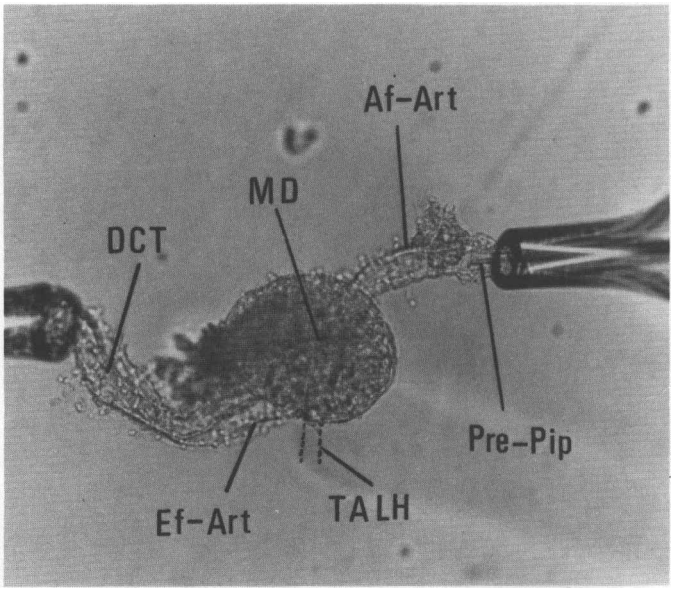

Fig. 2. *Microperfused afferent arteriole with attached macula densa.* Broken lines indicate a segment of the TALH which was removed previously. Abbreviations are the same as in Figure 1.

Images of the afferent arteriole were displayed at magnifications up to 1,980× and recorded with a video system consisting of a camera adaptor with a 3.3× photo-eyepiece, black-and-white charge-coupled camera (model NC 70; Dage-MTI, Inc., Michigan, Indiana, USA), monitor (model BWM 112; Javelin Electronics Inc., Torrance, California, USA), and video recorder (Sony, Tokyo, Japan). Since changes in arteriolar diameter were always seen at the distal end, the diameter at this point was measured with an image-analysis system (Fryer, Carpentersville, Illinois, USA).

Results

Microperfusion of both the afferent arteriole and macula densa was attempted in 14 rabbits and successfully completed in 10 (success rate 71%). Figure 2 shows an example of the preparation. Of four unsuccessful experiments, two were due to inability to either microdissect the afferent arteriole with at-

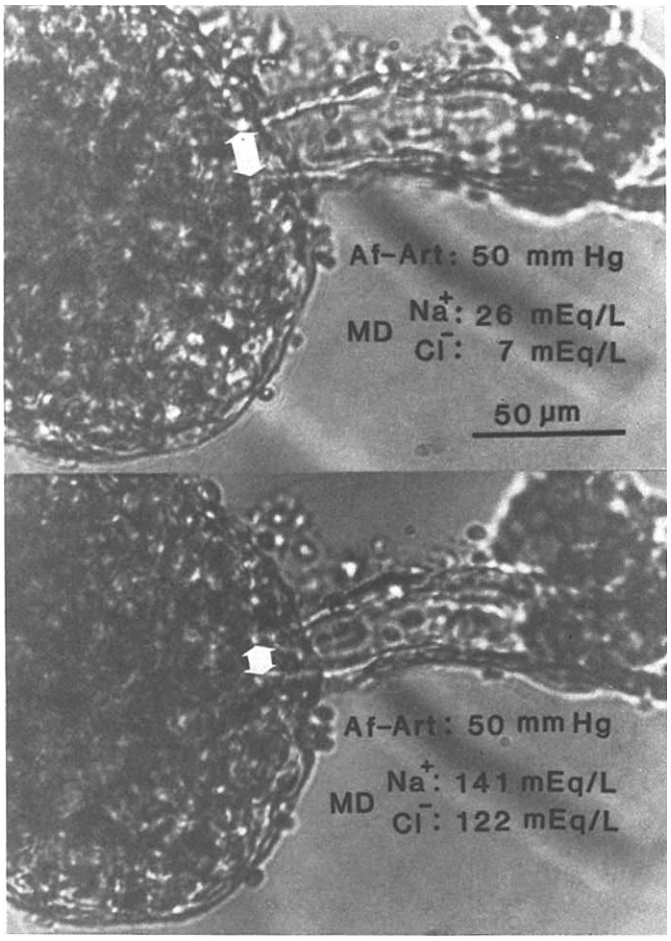

Fig. 3. *Constriction of the afferent arteriole induced by a high NaCl concentration at the macula densa.* Since the focus was on the afferent arteriole, the macula densa cannot be seen clearly.

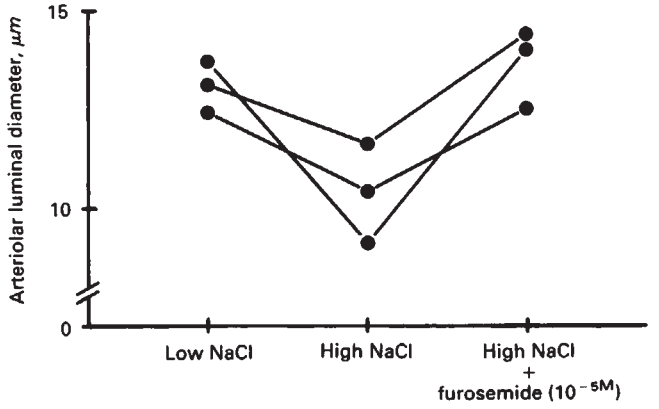

Fig. 4. *Constriction of the afferent arteriole induced by a high NaCl concentration at the macula densa and its blockade by furosemide.*

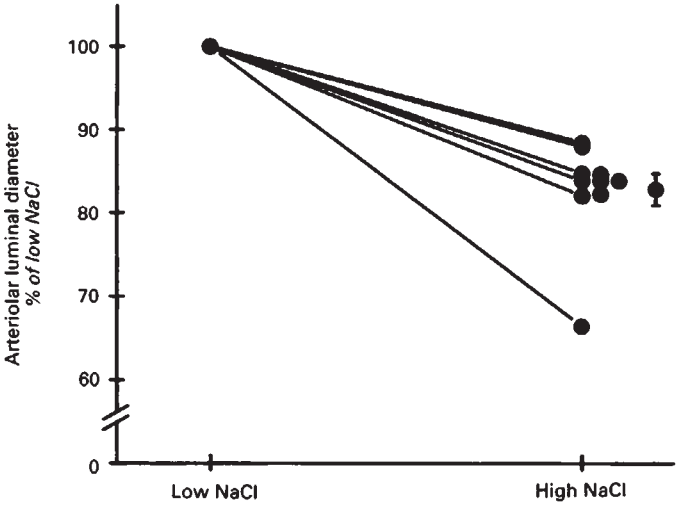

Fig. 5. *Influence of the NaCl concentration of the macula densa perfusate on the luminal diameter of the afferent arteriole.*

tached macula densa segment or cannulate the arteriole, and the other two were due to detachment of the endothelium during advancement of the perfusion pipette. In the cases of detachment, the perfusion pipette entered the space between the endothelium and vascular smooth muscle cells (pseudolumen), and the study was not carried further.

In seven experiments, we examined whether altering the NaCl concentration of the macula densa perfusate affects the diameter of the microperfused afferent arteriole. After equilibration, the macula densa was perfused with two solutions having different NaCl concentrations (high-NaCl and low-NaCl), while the arteriolar perfusion pressure was maintained at 50 mm Hg. The high-NaCl solution was a modified Krebs-Ringer bicarbonate buffer used during equilibration (Na^+, 144 mEq/liter and Cl^-, 122 mEq/liter). The low-NaCl solution was identical to the high-NaCl solution except that Na^+ and Cl^- were 26 and 6 mEq/liter, respectively (total osmolality, 88 mOsm/kg). The macula densa was first perfused with the low-NaCl solution for 15 minutes (control period), after which the perfusate was changed to the high-NaCl solution for the next 15-minute (experimental) period. Following this, the macula densa was again perfused with the low-NaCl solution for 15 minutes (recovery period). When the macula densa perfusate was changed from the low- to the high-NaCl solution, the afferent arteriole became constricted within 5 seconds; the strongest constriction was observed always at the most distal segment (Fig. 3). The luminal diameter measured at this segment decreased from 15.4 ± 1.8 to 13.0 ± 1.6 µm (mean ± SEM; $P < 0.0001$, paired t-test), and upon restoration of the low-NaCl solution it returned to 15.9 ± 1.9 µm, which is not significantly different from baseline ($P > 0.29$, paired t-test).

In three experiments, we tested the effect of furosemide, a known inhibitor of TGF. The macula densa was perfused with the low-NaCl solution for the first period and the high-NaCl solution for the second period. During the third period, the macula densa was perfused with the high-NaCl solution containing furosemide at 10^{-5} M. As shown in Figure 4, furosemide completely blocked the vasoconstriction induced by the high-NaCl solution.

Figure 5 compares the diameter during the first (low-NaCl) and second (high-NaCl) periods in all the above ten experiments, taking the diameter obtained with the low-NaCl solution

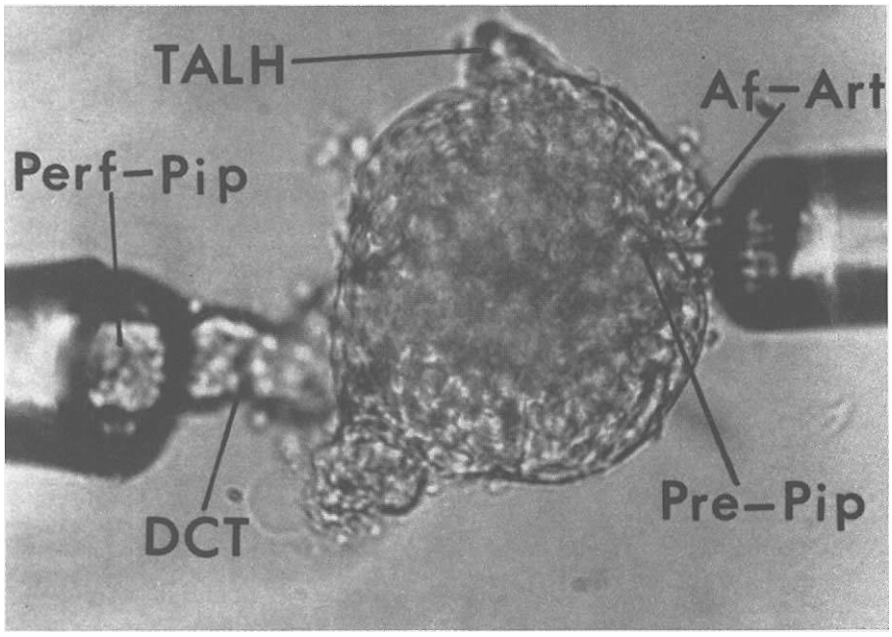

Fig. 6. *Microperfusion of both the glomerulus and macula densa with a pressure pipette in the glomerular capillary.* We performed two time control experiments in which the glomerulus was perfused at 50 mm Hg and the macula densa with a low-NaCl solution throughout the experiment (success rate 100%). Glomerular volume calculated from the area of the tuft was 1.3 and 1.2 $\times 10^{-3}$ mm^3 during the first 15-minute period, which was not altered by mock changes of the macula densa perfusate and remained stable during the second and third periods.

as 100%. The high-NaCl solution uniformly decreased the luminal diameter by an average of 17.2 ± 1.9% ($P < 0.0001$, one sample t-test).

Discussion

Microdissection and microperfusion techniques have proven to be useful for the study of the macula densa. Using microdissected and non-perfused rabbit afferent arterioles alone and with macula densa attached, we have shown that the macula densa inhibits renin release when incubated in an isotonic solution (medium 199), which may be mediated through its endogenous adenosine production [4, 5]. It has also been shown that macula densa-mediated renin release [8] as well as the electrophysiological properties of the macula densa cells [9] can be studied in the microperfused macula densa segment with the non-perfused glomerulus attached. In this report, we have demonstrated that it is feasible to study the hemodynamic function of the JGA directly in vitro. Our results show that increasing the NaCl concentration of the macula densa perfusate induces immediate vasoconstriction in the terminal segment of the afferent arteriole, and that this constriction is blocked by furosemide. In our preparation, there are no systemic hemodynamic or hormonal influences, nor the influence of transport by the tubular segments other than the macula densa. Therefore, our preparation is both viable and suitable for the study of macula densa-mediated glomerular hemodynamics.

Recently, the in vitro perfused juxtamedullary nephron preparation developed by Casellas, Carmines and Navar [10] was used to study renal autoregulation and TGF [11, 12]. These studies have shown that increasing the perfusion pressure or injecting a synthetic solution into the tubular lumen close to the macula densa constricts the afferent arteriole. Our results are consistent with these observations and strongly suggest that the macula densa is an anatomical site sensing the tubular signal, while the distal afferent arteriole is a site of the vascular response.

In general, this study is consistent with the observations obtained from numerous in vivo micropuncture studies, which in turn support the soundness of our preparation. This preparation has some distinctive features, for example: 1) it allows us to control the perfusion pressure of the afferent arteriole, thereby eliminating the hemodynamic influence of upstream renal vasculature, such as the interlobular arteries, which have been shown to exhibit a strong myogenic response [11]; 2) since it has only a short tubular segment contiguous with the macula densa (~150 μm), the composition of the luminal fluid at the macula densa can be controlled much more closely than it could be in vivo; and 3) unlike other preparations, it allows direct observation of vascular segments overlapped by the glomerulus and measurement of glomerular size.

Although measurement of efferent arteriolar diameter and glomerular size is feasible, we did not attempt it in this preparation, since they would always be under the hemodynamic influence of the afferent arteriole. For instance, when the afferent arteriole constricts in response to tubular signal at the macula densa, glomerular size and efferent arteriolar diameter may secondarily decrease independently of the tubular signal. Therefore, in order to study the individual response of the glomerulus or efferent arteriole, their perfusion pressure should be controlled. The approach employed in this study may potentially be useful for such a purpose. As shown in Figure 6, it is feasible to cut the afferent arteriole short and perfuse it with a pressure pipette placed in the glomerular capillary just beyond the distal end of the arteriole. This allows us to control glomerular perfusion pressure without hemodynamic influences of the afferent arteriole and study the response of the glomerulus per se to tubular signal. To study the response of the efferent arteriole, it may be microperfused in a retrograde direction at a constant pressure, using the same method as described for the afferent arteriole.

Our preparation could be used for other studies as well. Since it is feasible to microdissect both the midcortical and juxtamed-

ullary afferent arterioles [13] which are long enough to be cannulated, our preparation can be used to study the heterogeneity of macula densa-mediated glomerular hemodynamics among different nephron populations. Furthermore, since our preparation is an isolated JGA, we can manipulate various paracrine hormones much more effectively than we could in vivo. Therefore, this preparation will be useful to study mediator(s) and/or modulator(s) of the mechanism by which the macula densa controls glomerular hemodynamics.

A disadvantage of this preparation is its technical difficulty. Several months of training may be required to master the techniques of microdissecting the afferent arteriole with macula densa segment attached and preparing the various pipettes; among them, the most difficult part may be microdissection. However, once all the techniques are established, the success rate can be reasonably high, as 71% in this study.

In summary, this report presents a novel in vitro preparation to study the hemodynamic function of the JGA directly, and also provides direct evidence that the JGA plays a role in the control of glomerular hemodynamics.

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